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Mutat Res. Author manuscript; available in PMC 2010 March 9.

Published in final edited form as:
Mutat Res. 2009 March 9; 662(1-2): 84–87. doi:10.1016/j.mrfmmm.2008.11.012.

TREX2 Exonuclease Defective Cells Exhibit Double-Strand Breaks and Chromosomal Fragments but Not Robertsonian Translocations

Lavinia C. Dumitrache, **Lingchuan Hu**, and **Paul Hasty**
Department of Molecular Medicine/Institute of Biotechnology, The University of Texas Health Science Center at San Antonio, 15355 Lambda Drive, San Antonio, Texas 78245-3207, USA

Abstract

TREX2 is a 3′→5′ exonuclease that binds to DNA and removes 3′ mismatched nucleotides. By an *in vitro* structure function analysis, we found a single amino acid change (H188A) completely ablates exonuclease activity and impairs DNA binding by about 60% while another change (R167A) impairs DNA binding by about 85% without impacting exonuclease activity. For a biological analysis, we generated $trex2^{null}$ cells by deleting the entire *Trex2* coding sequences in mouse embryonic stem (ES) cells. We found Trex2 deletion caused high levels of Robertsonian translocations (RbTs) showing Trex2 is important for chromosomal maintenance. Here we evaluate the exonuclease and DNA binding domains by expressing in $trex2^{null}$ cells coding sequences for wild type human *TREX2* ($Trex2^{hTX2}$) or human *TREX2* with the H188A change ($Trex2^{H188A}$) or the R167A change ($Trex2^{R167A}$). These cDNAs are positioned adjacent to the mouse *Trex2* promoter by Cre-mediated knock-in. By observing metaphase spreads, we found $Trex2^{H188A}$ cells exhibited high levels of double-strand breaks (DSBs) and chromosomal fragments. Therefore, TREX2 may suppress spontaneous DSBs or exonuclease defective TREX2 may induce them in a dominate-negative manner. We also found $Trex2^{hTX2}$, $hTrex2^{H188A}$ and $hTrex2^{R167A}$ cells did not exhibit RbTs. Thus, neither the exonuclease nor DNA binding domains suppress RbTs suggesting TREX2 possesses additional biochemical activities.

Keywords

TREX2; exonuclease; double-strand breaks; Robertsonian translocations; genomic stability

1. Introduction

The mammalian Three prime Repair Exonuclease (TREX) proteins, TREX1 and TREX2 are homologous to the proofreading exonuclease in bacterial DNA polymerases important for postreplication repair [1–4]. *In vitro*, both function as homodimers, bind to DNA and effectively remove 3′ mismatched sequences via their 3′→5′ exonuclease activity [5]. To better understand biological function, *Trex1* was mutated in mice; however, surprisingly $trex1^{-/-}$ mice did not show genomic instability as would be expected for defective postreplication repair but instead died from cardiomyopathy caused by inflammatory myocarditis [6]. This

Corresponding author: Paul Hasty, Telephone: (210) 567-7278, Fax: (210) 567-7277, Email: E-mail: hastye@uthscsa.edu.

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unexpected phenotype suggested that TREX1 is not directly involved in DNA repair despite its sequence homology to exonucleases and its exonuclease activity. To help explain this surprising phenotype, TREX1 was later shown to process cytosolic DNA that may arise from endogenous retroelements or aberrant replication intermediates [7,8]. Failure to do so leads to the accumulation of cytosolic DNA that ultimately induces a pathological autoimmune response designed to defend against viruses or a chromic DNA damage checkpoint. To support these findings, TREX1 mutations cause a variety of autoimmune disorders in humans [9,10]. In addition, TREX1 is apart of the Granzyme A mediated cell death pathway since it binds to the SET complex and degrades nuclear DNA in concert with the endonuclease NM23-H1 [11]. Thus, TREX1 performs several functions, but does not appear to be important for DNA repair while much less is known about TREX2 biological function.

To better understand TREX2 biological function, we generated *trex2^null^* mouse ES cells by deleting all the known *Trex2* coding sequences via gene targeting [12]. We found *trex2^null^* cells exhibited high levels of Robertsonian translocations (RbTs). RbTs are chromosome rearrangements involving centric fusion of two acrocentric chromosomes to form a single metacentric chromosome that results from deletion of the p arms from both chromosomes [13,14]. RbTs may influence speciation [15,16] and may increase cancer risk [17], spontaneous abortions [18] and male infertility [19]. Thus, Trex2 maintains chromosomal integrity but we do not know if the exonuclease and DNA binding activities are important for suppressing RbTs.

TREX2 works as a homodimer to bind to DNA and remove 3′ mismatched sequences. Single amino acid mutations were made in TREX2 to separate DNA binding from exonuclease activity [20–22]. The H188A alteration completely ablates exonuclease activity but also impairs DNA binding by about 60% while the R167A change impairs DNA binding by about 85% without diminishing exonuclease activity [21]. Therefore, these functions may be genetically separated, at least in part, yet the biological significance of these activities is not known.

Here we report the impact human wild type TREX2 and human TREX2 mutated in the exonuclease domain or DNA binding domain has on mouse ES cells. We find Trex2-deletion mildly increases the level of spontaneous chromosomal DSBs and fragments. This phenotype is rescued by expression of wild type human TREX2 [21] and TREX2 mutated in the DNA binding domain. However, expression of TREX2 mutated in the exonuclease domain resulted in a further increase in chromosomal breaks suggesting a dominant negative phenotype. Surprisingly, expression of wild type TREX2 and either mutant prohibited the formation of RbT's, suggesting that TREX2 possesses functions other than DNA binding and exonuclease activity that are important for genomic stability.

2. Materials and Methods

2.1. Cell culture conditions

AB2.2 ES cells were maintained in M15 [high glucose DMEM supplemented with 15% fetal bovine serum, 100μM β-mercaptoethanol, 1 mM glutathione, 3 mg/ml penicillin, 5 mg/ml streptomycin, 1000 U/ml ESGRO (LIF)] and grown on plates with 2.5×10^6 γ-irradiated murine embryonic fibroblasts (mitotically inactive feeders) seeded on 0.1% gelatin coated plastic at least the day before and grown in 5% CO_2 in a 37°C incubator at atmospheric O_2.

2.2. Knock-in into ES cells

To integrate the human cDNA next to the mouse *Trex2* promoter, a Cre-mediated knock-in (CMKI) plasmid [23] with the short isoform of human cDNA [21] was transfected into *trex2^null-2^* cells that had been previously deleted for the 5′ half of the *HPRT* minigene [12]. 5 X 10^6 cells were co-electroporated with 20μg of CMKI plasmid and 10μg of a Cre-recombinase

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expression vector (pPGKcrepA) in a total of 800μl DPBS (Dulbecco's Phosphate Buffered Saline) using a Bio-Rad Gene Pulsar at 230V, 500μF. Then 200μl of the electroporation was seeded onto a 10cm feeder plate (primary embryonic fibroblasts mutated for *HPRT*). After 48 hours of transfection, a final concentration of 1 X HAT (1 mM sodium hypoxanthine, 4 μM aminopterin, and 160 μM thymindine) was added to the media. Eight-ten colonies were picked after 7–10 days of selection and expanded in HAT selection media to eliminate *HPRT* negative cells that survive by cross-feeding. These colonies were replica plated, and then one plate was frozen while the other plate was used to isolate DNA [24] for screening knock-in clones by genomic PCR.

2.3. Verification of knock-in

PCR verified knock-in by using Cre1 and hTX2Rev primers. PCR conditions: The forward primer (Cre 1: 5′ CCATGAGTCCTCTTTAAAGTG 3′) and reverse primer (hTX2Rev: 5′ CTGCAGCGTCCGCACCACG 3′) were used under these conditions: one cycle of 98°C 5 minutes followed by 35 cycles of 98 °C 1 minute', 63.5 °C 1 minute, 72 °C 1 minute 40 seconds followed by one cycle of 72 °C 10 minutes).

RT-PCR also verified knock-in by using primers specific to human *TREX2* (hTX2For, hTX2Rev). PCR was performed on RNA with and without reverse transcriptase (+/−) to ensure there is no DNA contamination. RT-PCR conditions: The forward primer (hTX2For: 5′ AAA AGA ATT CCC GCC ACC ATG TCC GAG GCACCCCGGGC 3′) and reverse primer (hTX2Rev2: 5′ CTGCAGCGTCCGCACCACG 3′) were used under these conditions: one cycle of 98 °C 5 minutes followed by 35 cycles of 98C 1 minute, 65°C 1 minute, 72 °C 25 seconds followed by one cycle of 72 °C 10 minutes).

2.4. Three-color FISH (Fluorescence in situ hybridization)

Treat cells with 10mgs colcemid for 4 hours then trypsinized cells. Slide preparation: Spin cells (1000 rpm), 10′ wash cells x2 in PBS (all PBS washes are pH 7.4 unless otherwise noted.). Resuspended pellet in 300mL 75mM KCl, dropwise, flicking tube. Incubate 37°C water bath, 15′. Add 300mL methanol/acetic acid (3:1 fixative), dropwise, flicking tube, spin 3000 rpm, 30″. Wash cells in 300mL 3:1 fixative, dropwise, flicking tube, spin @ 3000 rpm, 30″; rpt wash. Hybridization: Place slides in 70mM NaOH, 2′. Wash in PBS pH 8.5, 10 dips. Incubate 37 degrees, 5′ in the dark, in 500 μl/slide of 0.25mg/mL major satellite repeat (CY-3 5′ TGG AAT ATG GCG AGA AAA CTG AAA ATC ATG GAA AAT GAG A 3′) and telomeric [6-FAM 5′ $(CCCTAA)_7$ 3′] probes wash in PBS, 10 dips, coverslip in DAPI.

3. Results and Discussion

Here we evaluate the TREX2 exonuclease and DNA binding domains by introducing human *TREX2* coding sequences adjacent to the mouse *Trex2* promoter in *trex2^{null}* cells. Previously we generated *trex2^{null}* AB2.2 ES cells (Fig. 1A) by replacing the entire known mouse *Trex2* coding sequence [12] with a floxed hypoxanthine phosphoribosyltransferase (*HPRT*) minigene [25], referred to as *miniHPRT*. *MiniHPRT* is selected for expression in HAT (hypoxanthine, aminopterin, thymidine) or for absence of expression in 6-TG (6-thioguanine) and is composed of a phosphoglycerol kinase (PGK) promoter with an intron that separates exons 1–2 from exons 3–8. We modified *miniHPRT* by placing a right element (RE) mutant *loxP* [26] 5′ to the promoter and another RE mutant *loxP* in the intron [27]. After gene targeting, the 5′ half of *miniHPRT* was removed upon Cre-mediated recombination leaving behind a RE mutant *loxP* and the 3′ half of the minigene (Fig. 1B). These cells are now called *trex2^{null-2}*. The short isoform of wild type human *TREX2* cDNA [21] was targeted adjacent to the endogenous mouse *TREX2* promoter by a recently described protocol called Cre-mediated knock-in [23]. Three different cDNAs were used to generate ES cells with wild type human *TREX2* (*Trex2^{hTX2}*) or

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with the H188A mutation (*Trex2^{H188A}*) or the R167A mutation (*Trex2^{R167A}*) [21]. These cDNA sequences were cloned into a Cre-mediated knock-in (CMKI) plasmid that contains the 5′ half of *miniHPRT*. These CMKIs were cotransfected with a Cre recombinase expression plasmid into *trex2^{null-2}* cells. Knock-in clones may be isolated in HAT selection media since they regenerate *miniHPRT* (Fig. 1C). Multiple clones of each were isolated and confirmed by PCR and RT-PCR (Fig. 1D). Thus, we have multiple clones of mouse ES cells that express either wild type human *TREX2* or human *TREX2* with a single amino acid change (either H188A or R167A) using the mouse *Trex2* promoter.

We observed metaphase spreads derived from two clones of each knock-in by three-color FISH (Table 1, Fig. 2). These cells were stained with a telomeric probe (green), a major satellite repeat (MSR) probe in the pericentromere (red) and DAPI (blue) [28]. We found metaphase spreads derived from *trex2^{null-2}* cells exhibited a mild increase in chromosomal DSBs and fragments compared to AB2.2 clones (p=0.0023, Fisher's exact test) while metaphase spreads derived from *Trex2^{H188A}* cells exhibit much higher levels of DSBs and fragments compared to AB2.2 (p>0.0001), *trex2^{hTX2}* (p>0.0001) and *trex2^{null}* (p>0.0001) cells. These breaks are often located in or next to the pericentromere, a highly repetitive region that is composed of 6–8 Mb of tandem MSRs that undergo dynamic regulation [28]. However, *Trex2^{R167A}* cells exhibited the same levels of DSBs and fragments as compared to AB2.2 (p=1.0) and *Trex2^{hTX2}* (p=1.0) cells. Thus, *trex2^{null}* cells exhibit a mild (but significant) increase in DSBs and fragments while *Trex2^{H188A}* cells exhibit a much larger increase.

These data suggest TREX2 suppresses spontaneous chromosomal DSBs while TREX2^{H188A} induces chromosomal DSBs in a dominate-negative fashion. These observations suggest that TREX2 participates in a basic metabolic process to maintain chromosomes (in particular the pericentromere) and that TREX2-deletion leads to a low level of DSBs. Furthermore, exonuclease defective TREX2 may interfere with this metabolic process leading to a much larger increase in DSBs as compared to a simple deletion. Even though H188A causes a partial defect in DNA binding this dominant negative effect appears to be restricted to defective catalytic activity since R167A reduces DNA binding but does not increase DSBs. This observation also suggests that fully efficient DNA binding is not critical for suppressing these DSBs. At present, understanding this metabolic process is purely guesswork, but a tantalizing possibility is that TREX2 resolves anomalous DNA structures that may arise in highly repetitive DNA and failure to do so may result in broken chromosomes. Since *trex2^{null}* cells show elevated levels of RbTs [12], it would be interesting if the defective TREX2 variants can rescue this phenotype.

These metaphase spreads were also observed for RbTs. Previously we showed that about 16% of *trex2^{null}* metaphase spreads exhibited RbTs compared to about 0.25% of control spreads. We found that knock-in of the short human *TREX2* isoform by gene targeting rescued this phenotype [12]. We reproduced these data here, showing that Cre-mediated knock-in of the same cDNA also rescued this phenotype since no RbTs were observed out of a total of 73 metaphase spreads (Table 1). Interestingly we also find that *Trex2^{H188A}* and *Trex2^{R167A}* cells do not show RbTs: 0 RbTs out of 183 total metaphase spreads and 0 RbTs out of 145 total metaphase spreads, respectively. Thus, human TREX2 wild type (p>0.001, Fisher's exact test), H188A (p>0.001) and R167A (p>0.001) rescue the RbT phenotype showing that exonuclease activity and DNA binding are not important for suppressing this phenotype. This is surprising since there are no other known biochemical activities for TREX2 and suggests that TREX2 possesses other activities important for chromosomal maintenance. These data also suggest that the elevated DSBs observed in *trex2^{null}* and *Trex2^{H188A}* cells do not induce RbTs suggesting RbTs are not the result of repairing these breaks through an aberrant pathway.

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Here we evaluate the TREX2 exonuclease and DNA binding domains in mouse ES cells. By Cre-mediated knock-in, we introduced adjacent to the mouse *Trex2* promoter wild type human *TREX2* cDNA or human *TREX2* cDNA mutated in either the exonuclease domain (H188A) or the DNA binding domain (R167A). We find that $trex2^{null}$ cells display a mild increase in chromosomal DSBs while $Trex2^{H188A}$ cells exhibit a much higher level of these DSBs, usually in or near the pericentromere. These observations suggest TREX2 participates in a metabolic activity to maintain the pericentromere and perhaps other chromosomal regions. We also show that the exonuclease and DNA binding domains are not important for suppressing RbTs. Thus, TREX2 must possess other biochemical functions that suppress RbTs. These data suggest that TREX2 is important for maintaining chromosomal stability through multiple mechanisms.

Acknowledgements

We are grateful to Mr. Sergio Lopez for statistical analysis (The Department of Epidemiology and Biostatistics, The University of Texas Health Science Center at San Antonio). This work was supported by 3P30 CA054174-16S2, UO1 ES11044, 1 RO1 CA123203-01A1 to PH and T32 CA86800-03 to LCD.

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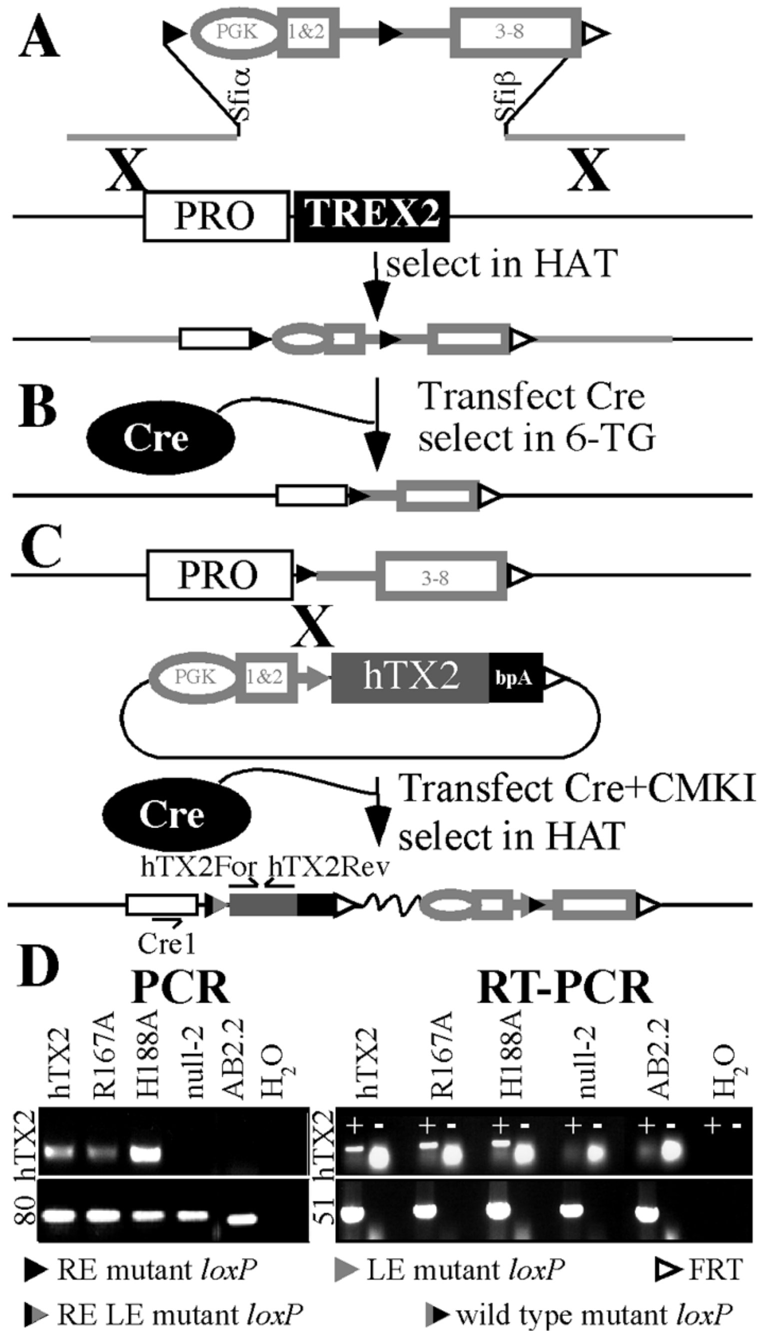

Fig. 1.

Knock-in of human *TREX2* cDNA variants. (A) The *HPRT* minigene, expressed by the PGK promoter [25,29] is used for selection and contains exons 1 and 2 (box labeled 1&2), exons 3–8 + polyadenylation sequences (box labeled 3–8) separated by an intron (straight line). Select for minigene expression in HAT. A RE mutant *loxP* (black arrow head) [26] is 5′ to PGK and another RE mutant *loxP* is in the intron. An FRT (open arrow) is located 3′ to *miniHPRT*. Upon targeting the entire known mouse *TREX2* open reading frame (rectangle) is deleted as previously described [12]; this sequence corresponds to the human short isoform [21]. PRO, mouse *Trex2* promoter. (B) Removal of the 5′ half of *miniHPRT* by Cre recombination as previously described [12]. (C) Knock-in of the short isoform of human *TREX2* cDNA (hTX2).

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A Cre-mediated knock-in plasmid is cotransfected along with a Cre-expression plasmid. The knock-in vector contains the 5′ half of *miniHPRT*, a left element (LE) mutant loxP (Grey arrow head) and the cDNA with bovine growth hormone polyadenylation sequences as previously described [23]. Cells are selected in HAT for restoration of *miniHPRT*. The knock-in corrects *miniHPRT*, generates an RE LE mutant loxP (left, black grey arrow), a wild type loxP (right, grey black arrow) and places the cDNA adjacent to the mouse TREX2 promoter. (D) Verification of knock-in. Due to the stringent selection, all HAT resistant clones are positive for knock-in as verified by PCR (left) using Cre1 and hTX2Rev primers. Ku80 (80) was used as a loading control for PCR as previously described [12]. In addition, human *TREX2* expression is confirmed by RT-PCR (right) using primers specific to human *TREX2* (hTX2For, hTX2Rev). Note the primers are specific for human *TREX2* since mouse *Trex2* is not amplified in the AB2.2 control. PCR was performed on RNA with and without reverse transcriptase (+/−) to ensure there is no DNA contamination. *Rad51* (51) was used as loading control for RT-PCR as previously described [12].

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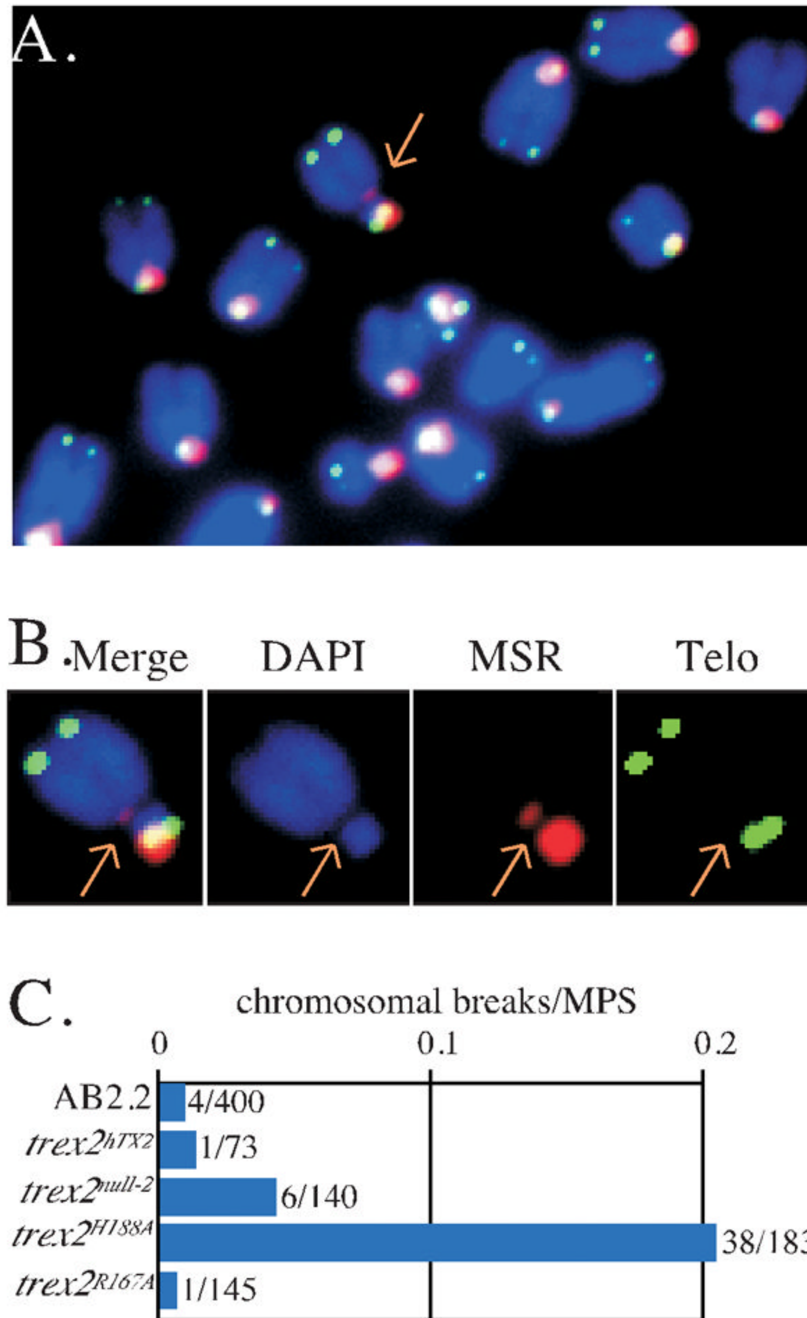

Fig. 2.

Trex2^{H188A} cells exhibit chromosome DSBs and fragments. Chromosomes are stained with DAPI (blue), a telomeric probe (green) and a MSR probe from the pericentromere (red). In mice the short arms are very small causing the telomeric and MSR probes to overlap resulting in a bleached yellow dot. (A) Metaphase spread with a chromosome DSB/fragment (arrow). (B) Enlargement of the chromosome with a DSB. Note the DSB occurs at the junction of the long arms and the pericentromere such that some of the pericentromere is still attached to the long arms. (C) Graph that shows the *Trex2*^{H188A} cells exhibit a greater number of chromosome DSBs compared to AB2.2, *trex2*^{null}, *Trex2*^{hTX2} and *Trex2*^{R167A} cells. The numbers for both clones shown in Table 1 is averaged.

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Table 1

Metaphase Spread Summary

		MPS	DSB	% DSB	1 RbT	2 RbT	total RbT	% RbT
1.	AB2.2	400	4	1.0	1	0	1	0.25
2.	*trex*2^{null}(2E1)	70	3	4.0	12	0	12	17.14
3.	*trex*2^{null}(2F7)	70	3	4.0	9	1	11	15.71
4.	*trex*2^{hTX2}(cl-1)	69	1	1.4	0	0	0	0
5.	*trex*2^{hTX2}(cl-2)	4	0	0	0	0	0	0
6.	*trex*2^{R167A}(cl-1)	76	1	1.3	0	0	0	0
7.	*trex*2^{R167A}(cl-2)	69	1	1.4	1	0	0	0
8.	*trex*2^{H188A}(cl-1)	84	12	14.3	1	0	0	0
9.	*trex*2^{H188A}(cl-2)	99	26	26.3	0	0	0	0

MPS: the total number of metaphase spreads observed.

1RbT: the number of metaphase spreads with one RbT.

2RbT: the number of metaphase spreads with two RbTs.